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ORIGINAL ARTICLE

Anti-fertility effects of fractions from *Carica papaya* (Pawpaw) Linn. methanol root extract in male Wistar rats

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Abstract The methanol root extracts of *Carica papaya* (Pawpaw) are used in eastern Nigeria for the treatment of malaria, hepatitis and jaundice. The aim of this study was to investigate the effects of the fractions isolated from *C. papaya* methanol root extract on fertility in male Wistar rats using sperm counts, percentage defective sperm cells (morphology), biochemical and hormonal assays as biomarkers. The roots of *C. papaya* were extracted using 80% methanol for 72 h. Oral acute toxicity study was done with the crude extract for 24 h. The extract was fractionated by column chromatography using petroleum ether, chloroform, ethyl acetate and methanol. The petroleum ether fraction was further fractionated on preparative TLC using ethyl acetate–methanol solvent systems to isolate CPFE 1, CPFE 2 and CPFM 1. The 3 fractions (75 mg/kg) were used to treat male Wistar rats orally for 60 days. Animals were euthanized and testes collected, homogenized and used for sperm count and motility. Plasma and serum were used to assay biochemical parameters including aspartate aminotransferase (AST), blood urea nitrogen (BUN), total bilirubin (TB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), triglycerides, hormones (LH and FSH). Histopathological study of the testes, kidney, heart and liver were conducted. Acute toxicity result showed that *C. papaya* root extract produced no mortalities at the dose of 2000 mg/kg but induced CNS-related symptoms as well as diuresis. The fractions significantly ($P < 0.01$) produced decreases in sperm counts and increased the percentage of defective sperm cells. There were significant ($P < 0.05$) increases in aspartate aminotransferase (AST) and blood urea nitrogen (BUN). Histopathological studies showed mild kidney and cardiac hyperaemia, slight

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hepatic degeneration and severe necrosis of the germinal epithelium of the testes. This study calls for some level of caution in the use of these roots and its extracts/fractions in traditional medicine for treating diseases. On the other hand, it could be a good source of drug for birth control.

1. Introduction

Nigeria, within a few years will be a leading country with the highest population growth in Africa. Since the population is tremendously on the increase, economists have forecast that this may drastically affect the economic growth. Family planning has been promoted through several methods of contraception, although these contraceptives have various side effects produced by their steroid content (Bingel and Benoit, 1973). Therefore, there is a need for drugs which are effective with lesser side effects.

Carica papaya (Pawpaw) is the sole species in the genus *Carica* of the plant family Caricaceae. Originally from Southern Mexico, Central America and Northern South America, the papaya is now cultivated in most countries with tropical climate like Nigeria (Oyekunle and Omope, 2010). *C. papaya* is known as okwulu bekee by the Igbo, Ibepe by the Yoruba and kawuse by the Hausa tribes of Nigeria. It is a fast growing semi-woody tree of about 6 m tall, stem normally not branched, and becoming hollow with age; male and female flowers usually grow on separate plants, reaching maturity within a year and living for 5–6 years only. It is widely cultivated throughout West Africa around habitations in the forest zone (Burkill, 1985).

Different parts of *C. papaya* plant are attributed with different medicinal values. For example, in African traditional medicine, the boiled green leaves are combined with leaves of *Azadirachta indica*, *Cymbopogon citratus*, *Psidium guajava* and stem bark of *Alstonia boonei* boiled together and the hot infusion is drunk as one wine glass full thrice daily in the treatment of malaria (Gill, 1992). Its fresh leaves are also efficacious in the treatment of gonorrhea, syphilis and amoebic dysentery (Gill, 1992). The milky juice of the unripe fruit is a powerful abortifacient, anti-helminthic for roundworms, stomach disorders and enlargement of liver and spleen (Gill, 1992). The seeds are also effective as a vermifuge and in the treatment of hypertension, diabetes mellitus and hypercholesterolemia (Gill, 1992). Results from studies on biological activities of *C. papaya* parts, extracts and isolated compounds showed that the latex and root extracts inhibited *Candida albicans* while extracts of pulp and seeds showed bacteriostatic properties against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis*, and *Entamoeba histolytica*, *in vitro* (Emeruwa, 1982). Its root aqueous extract has been shown to have a purgative effect (Akah et al., 1997).

C. papaya latex has been shown to have activity against *C. albicans* (Giordani et al., 1996), *Heligmosomoides polygyrus* (Satrija et al., 1995), *Ascaris suum* and *Ascaridia galli* (Satrija et al., 1994). Aqueous extract of *C. papaya* roots have shown potential activity in the management of dengue fever (Nisar et al., 2011), antitumor and immune-modulatory activities (Otsuki et al., 2010), hepatitis and jaundice in children.

In reproduction, various extracts of *C. papaya* have been shown to have antifertility activity in male (Chinoy and Padman, 1996) and female rats (Chinoy et al., 1997). It is also reported to be an abortifacient and a lactogenic (Burkill, 1985). Aqueous extract of *C. papaya* leaf caused reduction in mean values of andrological parameters as a result of lesion of the seminiferous tubule epithelium (Oyekunle and Omope, 2010). The present study therefore is to determine the effects of fractions from the methanol root extract of *C. papaya* on some reproductive and biochemical parameters in male Wistar rats.

2. Materials and methods

2.1. Preparation of the extract and fractionation

Fresh *C. papaya* (Linn.) roots were obtained from Nsukka urban region in November 2012 and authenticated by Mr. A. Ozioko, a taxonomist with the Biodiversity Development Centre Program (BDCP), Nsukka Enugu State, Nigeria. The roots (2.5 kg) were chopped into pieces and air dried on a laboratory bench. This was pulverized into coarse powder. 1000 g of the dried pulverized roots was extracted by cold percolation in 80% methanol:water for 72 h with intermittent shaking every 2 h. This was filtered with Whatman No. 4 filter paper and dried using a rotary evaporator (Büchi, Switzerland) at 40 °C. The extract was loaded into a 4 cm×50 cm glass column pre-loaded with silica gel (Silica Gel 60–120 mesh, 60A, Oxford, India) and fractionated using petroleum ether, chloroform:ethyl acetate (8:2) and ethyl acetate:methanol (3:7) as eluents. Following partial purification, the petroleum ether fraction, which showed significant effects on sperm parameters was further subjected to ethyl acetate and methanol sub-fractionation using preparative thin layer chromatography (TLC). Three compounds were obtained from the sub-fraction viz. CPFE 1 and 2, and CPFM 1. These were used for anti-fertility assay described below.

2.2. Animals

Adult male Wistar rats weighing between 170 and 210 g were used for the study. They were kept in the animal house of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka under a well-ventilated environment. The animals were allowed free access to feed and water *ad libitum*. The use of the animals conformed with internationally accepted principles for laboratory animal use and care as documented in the European Community guidelines, Council Directive, 1986 (86/609/EEC), revised in Directive 2010/63/EU. The experimental protocols were approved by the Ethics Committee for Animal Experimentation, University of Nigeria, Nsukka and in accordance with Nigerian Federal Government legislation on Animal care. All animals, including the control

group, were humanely euthanized before the onset of severe clinical conditions.

2.3. Oral acute toxicity studies

Oral acute toxicity study was carried out according to the method described by Miller and Tainter (1944). Rats were divided into five groups (1–5) consisting of 6 rats per group. Group 1 was given distilled water (10 ml/kg) while groups 2, 3, 4 and 5 were separately given 500, 1000, 1500, and 2000 mg/kg of the extract respectively. Treatments were administered orally by gastric intubation. The animals were observed for 24 h post treatment for signs of toxicity and then 48 h for possible death.

2.4. Experimental procedures

Thirty-two adult male albino rats were allocated into four groups (1–4) of 8 rats in each group. Groups 1, 2 and 3 were given 75 mg/kg b.w. of the CPFE 1 and CPFE 2, and CPFM 1 respectively daily orally for 28 days. Group 4 (control) served as normal control and were given distilled water (10 ml/kg). The fractions were dissolved in 0.5% Tween 20. After 60 days, blood sample from each rat was collected through the ocular retrobulbar plexus under light ether anesthesia. Blood samples were centrifuged at 1000 rpm and serum was collected for biochemical analysis. Serum biochemical parameters including aspartate aminotransferase (AST), blood urea nitrogen (BUN), total bilirubin, alkaline phosphatase (ALP), alkaline aminotransferase (ALT), gamma glutamyl transferase (GGT), triglycerides (TRIG) were determined using standard techniques (Norbert, 1986). Animals, including the control groups, were humanely euthanized and their testes, kidney and liver isolated. Sperm samples were collected from caudal epididymis for sperm count and defect studies. The abdominal cavities were opened through a midline abdominal incision to expose the reproductive organs. The testes were excised and all fats trimmed off, blotted dried and weighed with a Metler top loading weighing balance. Testes volumes were measured by water displacement method. The mean value of the two testicles from each rat was regarded as one score.

2.4.1. Epididymal sperm concentration

Spermatozoa in the right epididymis were counted by a modified method of Yokoi and Mayi (2004). In this method, the epididymis was minced with anatomic scissors in 5 ml physiologic saline, placed in a rocker for 10 min. This was allowed to incubate at room temperature for 2 min, after which the supernatant fluid was diluted 1:100 with a solution containing 5 g sodium bicarbonate ($NaHCO_3$) in 1 ml formalin (35%). Total sperm number was determined by using a haemocytometer. Approximately 10 µl of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and was allowed to stand for 5 min. This was viewed under a light microscope. The sperm concentration was then calculated.

2.4.2. Sperm progressive motility

This was evaluated by an earlier method by Sonmez et al. (2005). Motility estimates were performed from 3 different fields in each sample. The mean of the 3 estimations was used as the final motility score. Samples for motility evaluation were stored at 25 °C.

2.4.3. Sperm morphology

Sperm Cell morphologies were examined and evaluated using a light microscope at ×400 magnification. Caudal sperms were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin (Sigma–Aldrich, Jos, Nigeria). Five hundred randomly selected sperm cells from each sample were scored for morphological abnormalities. In wet preparations, spermatozoa were as follows: (1) normal head and tail, (2) isolated heads, whether by the head was misshapen or not, (3) head-only defects, that is misshapen head with normal tail, (4) tail defects, that is normal head with abnormal tail or misshapen head with abnormal tail and (5) fused sperm, and was expressed as a percentage of morphologically abnormal sperm.

2.4.4. Histopathology

Tissue samples from liver, kidney and testis were fixed in 10% formal-saline for a minimum of 24 h. The samples were dehydrated by washing in ascending grades of ethanol, cleared with xylene, embedded in paraffin wax, sectioned with a microtome, stained with Hematoxylin and Eosin (H&E) and mounted on Canada balsam. All sections were examined under light microscope (×10, ×20 and ×40) magnification. Photographs of the lesions were taken with an Olympus photo microscope for observation and documentation of histopathologic lesions.

2.5. Statistical analysis

The data collected were statistically analyzed using one-way Analysis of variance (ANOVA) and Duncan New multiple range post hoc test, mean differences at $p < 0.05$ were considered significant.

3. Results

3.1. Extraction and fractionation of the plant material

C. papaya methanol root extract gave a yield of 18.7% w/w dry extract. The fractions yielded CPFE 1 (250.6 mg) and CPFE 2 (210.3 mg) and CPFM 1 (342.7 mg). The developed TLC plates were sprayed with a solution freshly prepared of 50 mg ferric chloride ($FeCl_3$) in a mixture of 90 ml water, 5 ml acetic acid and 5 ml sulfuric acid. After heating at 100 °C for 3–5 min, the sterol spots are indicated by a red–violet color suggesting they may be sterol or glycosides.

3.2. Acute toxicity study

C. papaya methanol root extract was safe in rats at the tested oral doses (500–2000 mg/kg). There was no mortality within the study period. However, there were behavioral changes such as depression, reduced motor activity and ataxia. There was also a slight increase in urine output.

Table 1 Effect of CPFE 1, CPFE 2 and CPFM 1 from *Carica papaya* methanol root extract on serum biochemical parameters in rats.

Treatment	Dose (mg/kg)	TB (mg/dL)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	GGT (IU/L)	BUN (mg/dL)	TRIG (mg/dL)
CPFE 1	75	0.41 ± 0.08	118.25 ± 13.32	25.21 ± 4.11*	21.42 ± 3.01*	98.77 ± 12.22	57.45 ± 6.83*	55.17 ± 6.33
CPFE 2	75	0.36 ± 0.12	110.36 ± 14.35	15.21 ± 4.31	27.41 ± 4.51	95.82 ± 13.34	48.31 ± 7.36	61.21 ± 2.24
CPFM 1	75	0.30 ± 0.06	114.52 ± 16.41	21.73 ± 4.42*	23.77 ± 4.31*	97.91 ± 16.14	53.44 ± 5.81*	58.11 ± 6.72
Normal Control		0.43 ± 0.06	112.68 ± 13.70	10.13 ± 4.41	26.71 ± 4.36	93.17 ± 11.23	46.33 ± 8.85	62.31 ± 6.12

Values are expressed as mean ± SE for each group.
Total bilirubin (TB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alkaline amino transferase (ALT), gamma glutamyl transferase (GGT), blood urea nitrogen (BUN), and triglycerides (TRIG).
* Significant difference at $p < 0.05$ compared to control.

3.3. Serum biochemical analysis

CPFE 1 and CPFM 1 at 75 mg/kg significantly ($p < 0.05$) increased aspartate aminotransferase (AST) values when compared with control. CPFE 1 also induced significant ($p < 0.05$) increase in blood urea nitrogen (BUN) at 75 mg/kg. There was no significant ($p > 0.05$) difference in total bilirubin, alkaline phosphatase (ALP), alkaline amino transferase (ALT), gamma glutamyl transferase (GGT), and triglycerides in rats that were given different doses of the extract and control (Table 1). There were also no significant ($p > 0.05$) changes in triglycerides values between fraction-treated and normal rats.

3.4. Sperm morphology

The results of the sperm count, sperm motility and sperm morphology showed that CPFE 1 and CPFM 1 at 75 mg/kg significantly ($p < 0.05$) induced testicular inflammation (Table 2). The animals treated with CPFE 1 and CPFM 1 at 75 mg/kg significantly showed reduced sperm count, sperm motility and sperm morphology compared to the normal control (Table 2).

4. Discussion

It has been found that anti-malarial drugs usually possess anti fertility side effects. Some of these compounds include chloroquine (Adeeko and Dada, 1998); *Azadirachta indica*, *Alstonia boonei* (Oze et al., 2007) and dihydroartemisinin (Nwanjo et al., 2007). Different extracts from different parts of *C. papaya* have been known to be used in the treatment of malaria (Tijani et al., 2008; Arise et al., 2012). The leaves and roots of *C. papaya* contain cyanogenic glucosides which form cyanide (Bennett et al., 1997; Ayoola and Adeyeye, 2010). Thus there is a possible relationship between its anti-malarial and anti-fertility activities.

C. papaya methanol root extract was safe orally in rats up to 2000 mg/kg. This is in line with the findings of Halim et al. (2011), who reported no mortalities using its aqueous extract. However, the behavioral changes seen such as depression, reduced motor activity, ataxia, could be due to the presence of cyanogenic glycosides in *C. papaya* (Bennett et al., 1997; Ayoola and Adeyeye, 2010). These glycosides have been known to have deleterious effects on the brain due to cytotoxic hypoxia, leading to various degrees of nervous signs (Braide and Anika, 2007). This may be related to the folkloric use of the plant as a muscle relaxant and sedative (Ellingwood, 1919), which has been validated (Gupta et al., 1990). *C. papaya* leaves have been reported to be used in cases of edema and as a diuretic (Burkill, 1985). This could explain the slight increase in urine output seen in the rats.

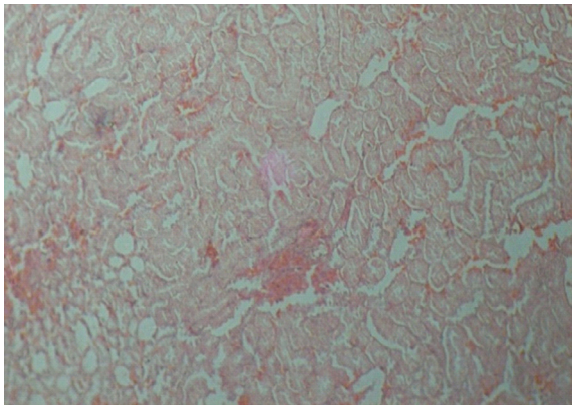

Figure 1 Kidney with mild hyperemia of glomerular and medullary vessels (staining H&E, magnification ×100).

Table 2 Effect of CPFE 1, CPFE 2 and CPFM 1 from *Carica papaya* methanol root extract on sperm count, motility and morphology in rats.

Groups/doses (mg/kg)	Sperm count (x10^6m/L)	% Sperm motility	Sperm morphology	
			Normal	Abnormal
CPFE 1 (75)	57.45 ± 5.36*	42.7 ± 1.72*	25.71 ± 2.12*	73.39 ± 2.74*
CPFE 2 (75)	85.71 ± 4.71*	81.36 ± 1.31	57.44 ± 3.78	41.56 ± 2.21*
CPFM 1 (75)	68.77 ± 4.88*	45.68 ± 1.17*	33.25 ± 2.16*	65.75 ± 3.27*
Normal control	142.1 ± 6.4	99.41 ± 1.15	93.81 ± 3.56	5.19 ± 3.42

* $p < 0.05$ different compared with normal control (ANOVA, LSD post hoc); $n = 8$.

The fractions increased the levels of AST and BUN in the rats. An increase in BUN is often associated with dehydration which can be caused by the diuresis observed in rats during the acute toxicity test. Increase in BUN could also be caused by excessive protein catabolism. This may be because *C. papaya* contains proteolytic enzymes papain and chymopapain (Brocklehurst and Salih, 1985) and glycosides; which cause increased proteolysis, resulting in the release of free amino acids as well as nitrogen and urea. The increased BUN could also presumably be due to lesions found in the kidney histopathology and this suggests that CPFE 1 and CPFM 1 may be nephrotoxic.

AST activity is usually high in the liver of all domestic animals and the serum activity is used routinely in all for evaluation of liver cell injury. However, AST activity is also high in the kidney, heart and skeletal muscle, so elevations in serum AST are considered less specific for liver diseases than ALT. Thus, the increased level of AST cannot be safely attributed to hepatic injury only. This is the reason for histopathological study of the heart (Fig. 2) and kidney (Fig. 1) which showed some mild hyperemia, while that of the liver (Fig. 3) showed mild hepatic degeneration. This explains the lack of significant difference in other liver function markers between rats given the fractions from *C. papaya* methanol root extract and the control. A previous study (Halim et al., 2011) using the aqueous extract of *C. papaya* did not bring about any significant changes in liver enzymes. The fractions, therefore, may not have severe toxic effects on the liver and heart.

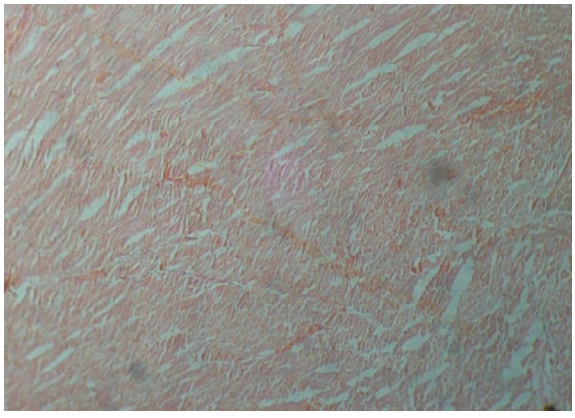

Figure 2 A mildly hyperemic muscle of the heart stained with H&E, magnification is ×100.

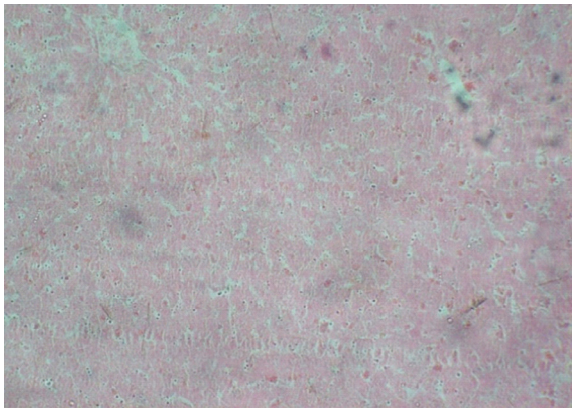

Figure 3 Normal liver stained with H&E.

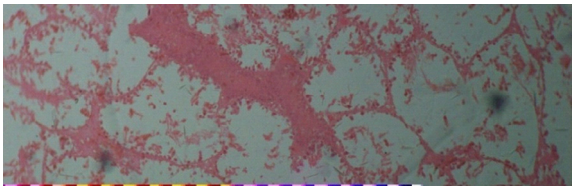

Figure 4 Testis having sloughing-off germinal epithelium.

CPFE 1, CPFE 2 and CPFM 1 from *C. papaya* methanol root extract produced significant ($P < 0.01$) reduction in spermatozoa count of rats. It also increased the percentage of sperm cells that are defective when compared to the normal control. The drastic decline in the rat sperm count and prevalence of a high percentage of defective spermatozoa at 75 mg/kg of CPFE 1 and CPFM 1 support the antifertility effect of the extract from the leaves (Ude and Nwaehujor, 2013). It also gives rationale for the use of *C. papaya* seeds in controlling overpopulation in Tilapia fish (*Oreochromis niloticus*) by fish farmers (Ekanem and Okoronkwo, 2003).

Histopathological study of testes showed marked necrotic lesions and sloughing off of the germinal epithelium in rats given CPFE 1 (Fig. 4). This may have been responsible for the low sperm count and the high percentage of defective sperm cells seen in rats in this group as compared to other groups. Some toxic constituents and adverse effects of the plant have also been reported. The major cyanogenic glycoside in *C. papaya* is (2R)-prunasin; small amounts of sambunigrin are also present (Seigler et al., 2002). A toxic substance called carpine is present in traces in pawpaw black seeds. In large quantities, carpine has been shown to cause paralysis, lower pulse rate and depress the nervous system (Wickersham and Novak, 2003). The crude *C. papaya* latex is a severe irritant and vesicant which can induce spasmodic contraction of the uterine muscles similar to oxytocin and prostaglandin F2α (Adebiyi et al., 2002). Pawpaw roots may contain antifertility properties in male rats and possibly man.

The exact mechanism by which the fractions reduced sperm count is not known, but it has been suggested that the compound papain may cross the blood testis barrier to exert harmful effects on control of the seminiferous tubules of the testes (Oyekunle and Omope, 2010). Many researches have reported the presence of proteins in both the sperm as well as the semen. Edwards et al. (1981) reported the presence of serum proteins (albumin, lactoferrin), glycoproteins – a kinase and prostatic. These proteins nourish the sperm cells. In rats given CPFE 1 and CPFM 1, the increased percentage of defective sperm cell could be due to the proteolytic action of the proteases in *C. papaya* papain and chymopapain or and also the steroidal glycosides. These enzymes and compounds may have hydrolyzed the semen proteins, making them unavailable for use by the sperm cells, thereby leading to malnutrition and defects seen in the cells.

5. Conclusion

The results obtained from this study calls for some level of caution in the use of these roots in traditional medicine for treat-

ment of diseases as it could be a good source of drug for birth control in males. Further work is on-going on *C. papaya* roots to determine the exact mechanism of action(s) for the observed changes in the biochemistry and morphology of the semen as well as identify the actual compound from the extract that possesses these antifertility properties.

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